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Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated

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Summary

Production of actinorhodin, a polyketide antibiotic made by *Streptomyces coelicolor* A3(2), normally occurs only in stationary-phase cultures. S1 nuclease protection experiments showed that transcription of *actII-ORF4*, the activator gene required for expression of the biosynthetic structural genes, increased dramatically during the transition from exponential to stationary phase. The increase in *actII-ORF4* expression was followed by transcription of the biosynthetic structural genes *actIII* and *actVI-ORF1*, and by the production of actinorhodin. The presence of *actII-ORF4* on a multicopy plasmid resulted in enhanced levels of *actII-ORF4* mRNA, and transcription of *actIII* and actinorhodin production during exponential growth, suggesting that actinorhodin synthesis in rapidly growing cultures is normally limited only by the availability of enough of the activator protein. *bldA*, which encodes a tRNA^{Leu}_{UUA} that is required for the efficient translation of a single UUA codon in the *actII-ORF4* mRNA, was transcribed throughout growth. Moreover, translational fusions of the 5' end of *actII-ORF4* that included the UUA codon to the *ermE* reporter gene demonstrated the presence of functional *bldA* tRNA in young, exponentially growing cultures and no increase in the efficiency of translation of UUA codons, relative to UUG codons, was observed during growth. The normal growth-phase-dependent production of actinorhodin in the liquid culture conditions used in these experiments appears to be mediated at the transcriptional level through activation of the *actII-ORF4* promoter.

Introduction

Antibiotic production in the Gram-positive prokaryotic

genus *Streptomyces* generally occurs in the stationary phase in liquid medium, and correlates temporally with the onset of morphological differentiation in surface-grown cultures (Demain *et al.*, 1983; Chater, 1989). Much progress has been made in elucidating the organization of antibiotic biosynthetic gene clusters in several different *Streptomyces* species (Hunter and Baumberg, 1989), and a number of pathway-specific regulatory genes have been identified that are required for the activation of their cognate biosynthetic structural genes (Distler *et al.*, 1987; Narva and Feitelson, 1990; Fernández-Moreno *et al.*, 1991; Raibaud *et al.*, 1991; Stutzman-Engwall *et al.*, 1992; Geistlich *et al.*, 1992). However, little is understood of the regulatory mechanisms that are involved in the expression of the activator genes.

In *Streptomyces coelicolor* A3(2), the most fully genetically characterized streptomycete, potential pleiotropic regulatory genes have been identified that are required for, or influence, the production of all four of the antibiotics (actinorhodin, undecylprodigiosin, methylenomycin, and a calcium-dependent antibiotic; Hopwood, 1988) known to be made by this strain. These genes fall into two classes: those that affect only antibiotic production (*absA* (Adamidis *et al.*, 1990) and *absB* (Champness *et al.*, 1990), *afsB* (Horinouchi *et al.*, 1983; Horinouchi *et al.*, 1989), *afsR* (Horinouchi *et al.*, 1990) and *abaA* (Fernández-Moreno *et al.*, 1992)), and those that affect both antibiotic production and morphological differentiation (*bldA–D* and *bldG–H*; Hopwood, 1988; Champness, 1988; Chater, 1989). The existence of these *bld* mutants suggests that morphological differentiation and antibiotic production share some elements of genetic control. Of particular note is *bldA*; this gene encodes a tRNA^{Leu}_{UUA} that is apparently the only tRNA species in *S. coelicolor* A3(2) that can efficiently translate the rare leucine codon UUA (Leskiw *et al.*, 1991b). Moreover, the majority (12/15) of UUA codons so far identified in streptomycetes are found in genes connected with antibiotic production or morphological differentiation that are expected to be expressed late in growth (Leskiw *et al.*, 1991a). In particular, *actII-ORF4*, the pathway-specific activator gene for actinorhodin biosynthesis, encodes an mRNA that contains a single UUA codon whose replacement by UUG led to a loss of *bldA*-dependence of actinorhodin production,

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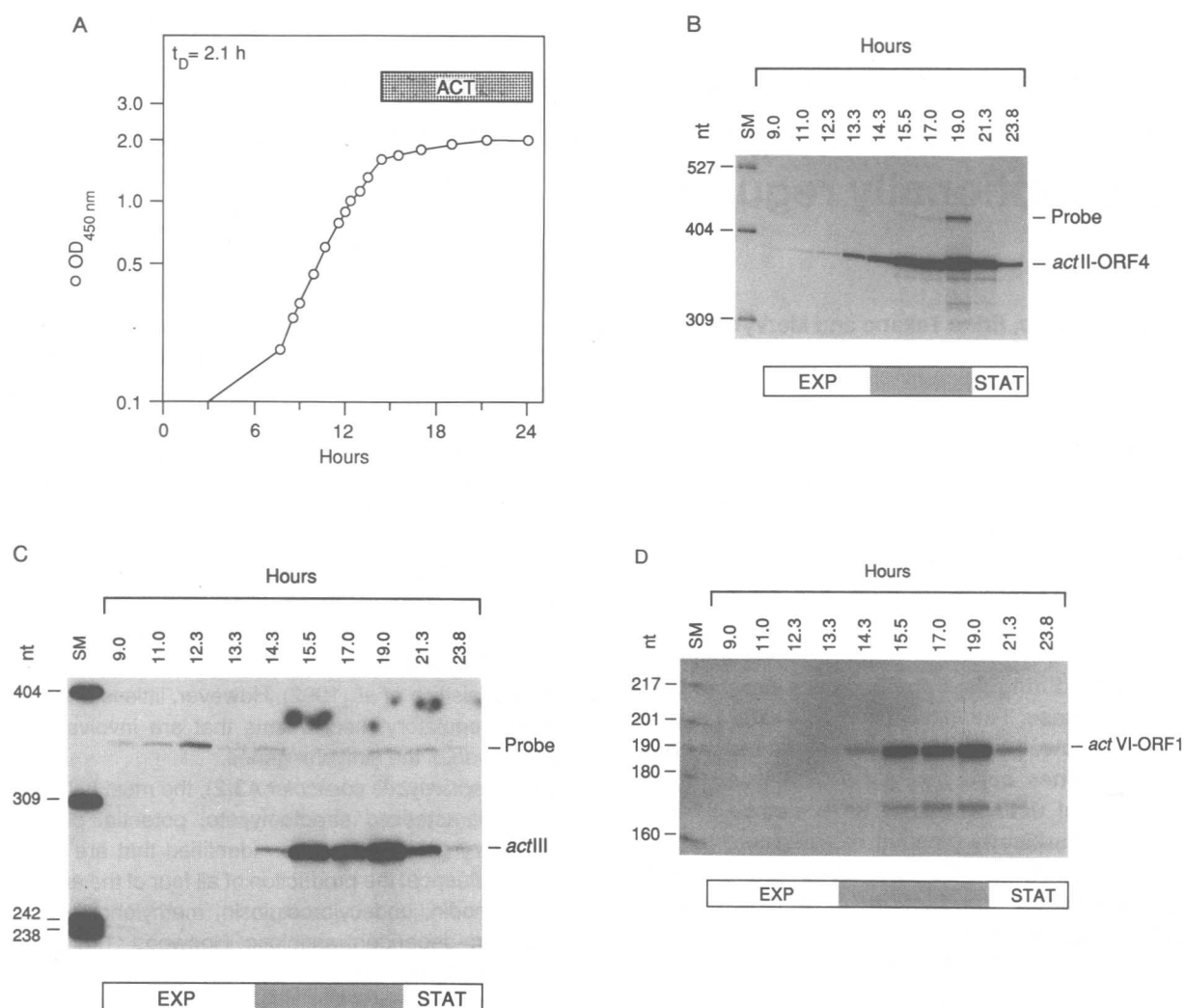


Fig. 1. A. Growth curve of *S. coelicolor* A3(2) strain M145 in casamino-acid-supplemented medium; t_D , doubling time (h); the shaded box labelled ACT indicates the presence of actinorhodin in the culture.

B, C and D. S1 nuclease mapping of *actII-ORF4* (B), *actIII* (C), and *actVI-ORF1* (D) transcripts in RNA samples obtained from the culture shown in (A) at the times indicated; EXP and STAT, exponential and stationary phases of growth, respectively; the shaded area between them indicates the transition phase; SM, end-labelled *Hpa*II-digested pBR322 size marker; Probe, the location of full-length probe. The lower band present in (D) may represent a second transcriptional start site for *actVI-ORF1*, RNA processing or degradation, or an artefact of the S1 nuclease mapping procedure.

prompting suggestions that this codon was the direct target for translational control of actinorhodin production by *bldA* (Fernández-Moreno *et al.*, 1991).

Here, we assess the temporal and growth-phase-dependent expression of *actII-ORF4*, the actinorhodin biosynthetic structural genes *actIII* (Hallam *et al.*, 1988) and *actVI-ORF1* (F. Malpartida, personal communication), and *bldA*, and provide evidence to suggest that *bldA* is not involved, under the conditions used, in the temporal regulation of actinorhodin production, which appears to occur at the transcriptional level.

Results

Actinorhodin gene expression is growth-phase-dependent

S. coelicolor A3(2) strain M145 grew in casamino-acid-supplemented minimal medium with a doubling time of 2.1 h and began the transition into stationary phase approximately 15 h after inoculation, whereupon actinorhodin appeared in the mycelium (Fig. 1A). S1 nuclease protection experiments with RNA from exponential and stationary phase cultures revealed *actII-*

ORF4 transcripts at a low level in exponential phase; their amount increased greatly at the end of exponential growth (Fig. 1B), with the highest levels occurring during the transition phase (the increase in the level of fully protected probe, which follows the increase in the level of the *actII*-ORF4 transcript, was not always observed, but may reflect transcriptional readthrough from *actII*-ORF3 (Fernández-Moreno *et al.*, 1991)). Transcripts for *actIII* and *actVI*-ORF1 were detected 13.3 h and 14.3 h after inoculation, respectively (Fig. 1, C and D), and increased in abundance as the culture entered stationary phase, following closely the increase in the level of *actII*-ORF4 mRNA.

The effect of extra copies of *actII*-ORF4 on the temporal expression of *actIII* and on actinorhodin production was assessed in M145 containing multicopy plasmid pIJ68. *actII*-ORF4 transcripts were readily detected in early exponential cultures and their level increased markedly in mid-exponential phase and then remained constant (Fig. 2A). A similar, but slightly delayed profile, was observed for the *actIII* transcripts (Fig. 2B) (a faint band was also observed in the earliest sample ($OD_{450nm} = 0.28$) after prolonged exposure). Actinorhodin production was first detected spectrophotometrically during mid to late exponential growth; the concentration in stationary-phase cultures was approximately 10 times that observed with M145.

Transcription of *bldA* occurs throughout growth in liquid culture

S1 nuclease protection experiments were performed with RNA from exponential- and stationary-phase cultures to assess the temporal pattern of *bldA* transcription (Fig. 3). The smallest protected fragment (approximately 80 nucleotides (nt) represents the 5' end of the mature

tRNA^{Leu}_{UUA} (Lawlor *et al.*, 1987); the other two bands were observed also either in *in vitro* transcription studies (the band of approximately 150 nt; (Lawlor, 1987) or sometimes in primer extension experiments (the band of approximately 100 nt; B. K. Leskiw, personal communication), and both may represent *in vivo* transcriptional start sites. Whereas the levels of the larger protected fragments declined with culture age (after an initial increase in the c. 100 nt fragment), the fragment representing the 5' end of the mature tRNA^{Leu}_{UUA} increased slightly during early exponential growth and then remained relatively constant. An essentially identical profile of *bldA* transcripts was observed using a richer medium (YEME; Hopwood *et al.*, 1985) and spores of *S. coelicolor* A3(2) M145 that had not been germinated prior to inoculation, suggesting that the profiles are typical of cultures grown in liquid medium.

To confirm that the signals observed represented *bldA*, and not cross-hybridization to related tRNA species, S1 nuclease protection experiments were carried out on RNA isolated from early and late exponential phase, and stationary-phase cultures of *S. coelicolor* A3(2) strains J1501 (*bldA*⁺) and J1681 ($\Delta bldA$); prolonged exposure revealed a faint signal with RNA from J1681, but a very strong one with RNA from J1501 (Fig. 3; only the results obtained with the stationary-phase cultures are shown), indicating that almost all of the signal observed in M145 represented *bldA* transcripts.

Translation of UUA codons in an *actII*-ORF4::*ermE* fusion transcript occurs throughout growth in liquid culture

The presence of *bldA* transcripts throughout growth did not necessarily reflect the presence of a functional *bldA* tRNA^{Leu}_{UUA}; perhaps the lack of 3' processing, charging by the appropriate amino acyl tRNA synthetase, or some

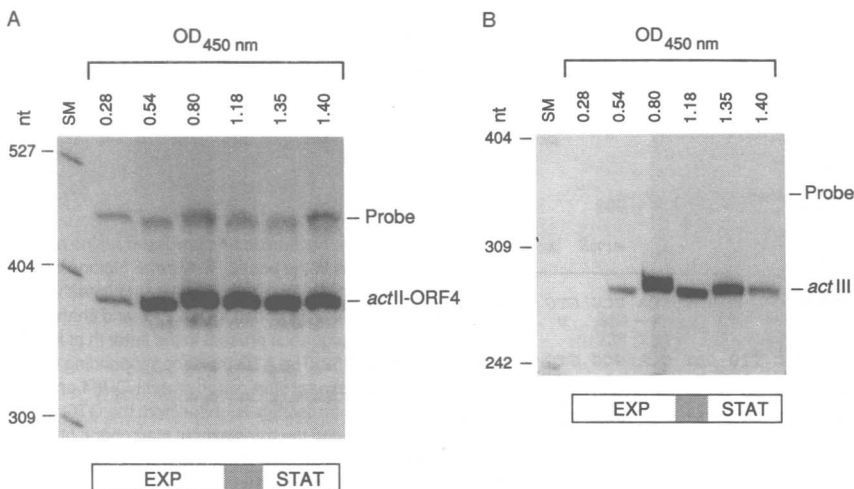


Fig. 2. A, B. S1 nuclease mapping of *actII*-ORF4 (A) and *actIII* (B) transcripts in RNA samples obtained from M145(pIJ68) cultures at the optical densities indicated. See the legend to Fig. 1 for other abbreviations.

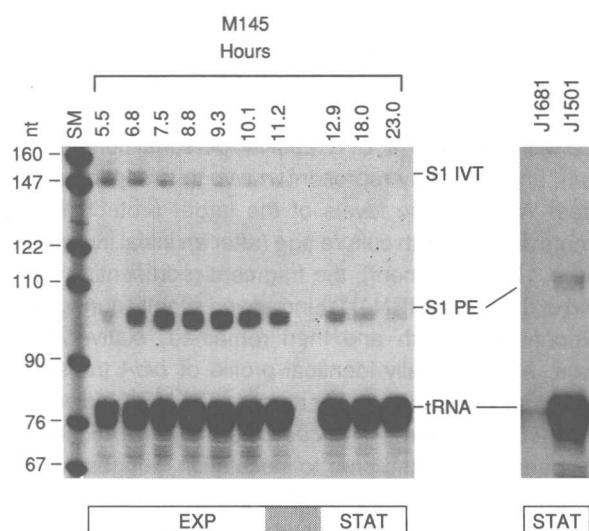


Fig. 3. S1 nuclease mapping of *bldA* transcripts in RNA samples prepared from *S. coelicolor* A3(2) strains M145, J1501 and J1681 ($\Delta bldA$). tRNA, protected fragment corresponding to the 5' end of the mature *bldA* tRNA; S1 PE and S1 IVT, S1-nuclease-resistant fragments corresponding to the 5' end of transcripts observed previously in primer extension experiments or in *in vitro* transcription studies, respectively. See the legend to Fig. 1 for other abbreviations.

other modification, prevented translation of UUA codons during exponential growth. To address this point, translational fusions were made between the 5' region of *actII*-ORF4, including the *actII*-ORF4 promoter and the unique UUA codon, and the *ermE* gene of *Saccharopolyspora erythraea*. *ermE*, which confers resistance to macrolide, lincosamide and streptogramin B-type antibiotics, encodes a 23S rRNA methylase (Uchiyama and Weisblum, 1985; Dhillon and Leadlay, 1990) for which polyclonal antibodies had been raised using oligopeptides as antigens. pIJ2250 contained the first five codons of *actII*-ORF4, with the fifth being the unique UUA codon; pIJ2251 differed in that the UUA codon had been changed to the synonymous leucine codon UUG, and pIJ2252 differed from pIJ2250 in containing additional UUA codons at positions six and seven (Fig. 4). Protein

and RNA samples were prepared from cultures containing the three plasmids during exponential growth and during the transition into stationary phase, and analysed by Western blotting and S1 nuclease mapping. The *ermE* methylase (with a predicted size of 43 kDa (EMBL nucleotide database accession number X51891), but which migrates on SDS-polyacrylamide gels against pre-stained markers with an apparent size of 45 kDa; M. J. Bibb, unpublished) was readily detected in extracts from a control culture, M145(pIJ427) (a pIJ486 derivative that contains the native *ermE*), but not in extracts from M145(pIJ486) (a negative control). The three constructions containing the translational fusions were expected to give a novel protein of 44.3 kDa; all three yielded a fusion protein larger than the native methylase. Moreover, the fusion protein was readily detected in early exponential growth and increased in amount as the culture entered the transition phase. While the amounts of fusion protein detected in extracts of M145 containing pIJ2250 or pIJ2251 were very similar, the level obtained from pIJ2252 was higher. To assess whether this difference, and the temporal increase in the level of the fusion proteins, reflected an increase in translational efficiency or an increase in the amount of transcript, RNA from culture samples corresponding in time to those used for protein extraction were monitored by S1 nuclease mapping (Fig. 5B). The fusion protein:fusion transcript ratio increased during growth approximately twofold for all three cultures. The level of fusion mRNA observed with pIJ2252, which contains three UUA codons, was notably higher than with the other two constructions (Fig. 5B).

Translation of actII-ORF4::ermE fusion transcripts containing UUA codons is bldA-dependent

The presence of *actII*-ORF4 on a multicopy plasmid restores actinorhodin production to *bldA* mutants (Pasantino *et al.*, 1991), presumably by a low level of mis-translation of the unique UUA codon by another tRNA via an imperfectly matched codon-anticodon interaction; increased levels of *actII*-ORF4 transcripts from the multicopy vector presumably allow for the synthesis of enough

	<i>actII</i> -ORF4										<i>ermE</i>				
pIJ2250	GCGCAG	<u>ATG</u>	AGA	TTC	AAC	<u>TTA</u>	TTC	TTC	TTG	<u>GAT</u>	CCA	GCG	GTG	-	<i>ermE</i>
		fM	R	F	N	L	F	F	L	D	P	A	V		
pIJ2251	GCGCAG	<u>ATG</u>	AGA	TTC	AAC	<u>TTG</u>	TTC	TTC	TTG	<u>GAT</u>	CCA	GCG	GTG	-	<i>ermE</i>
		fM	R	F	N	L	F	F	L	D	P	A	V		
pIJ2252	GCGCAG	<u>ATG</u>	AGA	TTC	AAC	<u>TTA</u>	<u>TTA</u>	<u>TTA</u>	TTG	<u>GAT</u>	CCA	GCG	GTG	-	<i>ermE</i>
		fM	R	F	N	L	L	L	L	D	P	A	V		

Fig. 4. The nucleotide and predicted amino acid sequences in the *actII*-ORF4::*ermE* fusions in pIJ2250–pIJ2252. Shaded triplets indicate the *actII*-ORF4 UUA codon in pIJ2250, and changes from the sequence present in the latter in pIJ2251 and pIJ2252. The ATG triplet corresponding to the translational start codon of *actII*-ORF4 and of the fusion transcripts is underlined; the GTG corresponding to the translational start codon of *ermE* is in italics and the *Bam*HI site used for cloning is in bold letters.

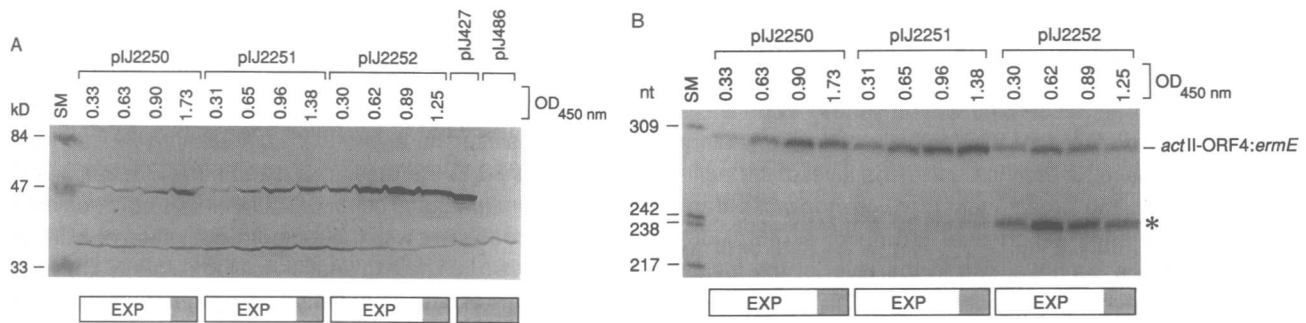


Fig. 5. A. Western analysis of cell extracts of *S. coelicolor* A3(2) strain M145 derivatives containing pIJ2250, pIJ2251, and pIJ2252, using antibodies against the *ermE* methylase. Cell extracts from M145 containing pIJ427 and pIJ486 were used as positive and negative controls, respectively; SM, size markers.

B. S1 nuclease mapping of *actII-ORF4::ermE* transcripts in RNA samples from the same cultures used in A. The asterisk indicates the protected fragment that results from cleavage by S1 nuclease at the region of mismatch between the probe (obtained by PCR using pIJ2250 as a template) and the fusion transcripts. The faint bands present at a similar position in the pIJ2250 and pIJ2251 tracks do not comigrate with this protected fragment upon prolonged electrophoresis and are present in all lanes; they may reflect an artefact of the S1 nuclease mapping procedure. See the legend to Fig. 1 for other abbreviations.

activator to elicit expression of the *act* structural genes. The same explanation might have applied to the results obtained with the *actII-ORF4::ermE* fusions, since they are also present on a high-copy-number plasmid. To assess whether the fusion protein detected by Western blotting in young cultures resulted from translation of the UUA codons by tRNA^{Leu}_{UUA}, and not by mis-translation, the three plasmids were introduced into the *S. coelicolor* A3(2) *bldA* mutant J1700, and the amount of fusion protein and fusion mRNA in exponential cultures was analysed. The fusion protein was detected only in extracts prepared from J1700(pIJ2251) (Fig. 6A), although the levels of fusion transcript detected in J1700(pIJ2250) and J1700(pIJ2251) were very similar (Fig. 6B). As noted for M145, the level of fusion mRNA with pIJ2252 was higher than with the other two constructions (Fig. 6B).

Discussion

Transcription of the activator gene *actII-ORF4* is clearly growth-phase-dependent in liquid medium, reaching a maximum during the transition from exponential to stationary phase. Transcription of *actIII* and *actVI-ORF1* occurs after the accumulation of almost maximal levels of the *actII-ORF4* transcript, suggesting that a threshold concentration of the activator is needed for transcription of the biosynthetic structural genes, a possibility noted by Hopwood *et al.* (1986). The presence of multiple copies of *actII-ORF4* leads to high levels of the *actII-ORF4* transcript; probably as a consequence, expression of *actIII* is enhanced and actinorhodin appears in early to mid-exponential growth, suggesting that the only limitation on actinorhodin production in rapidly growing cultures, under the

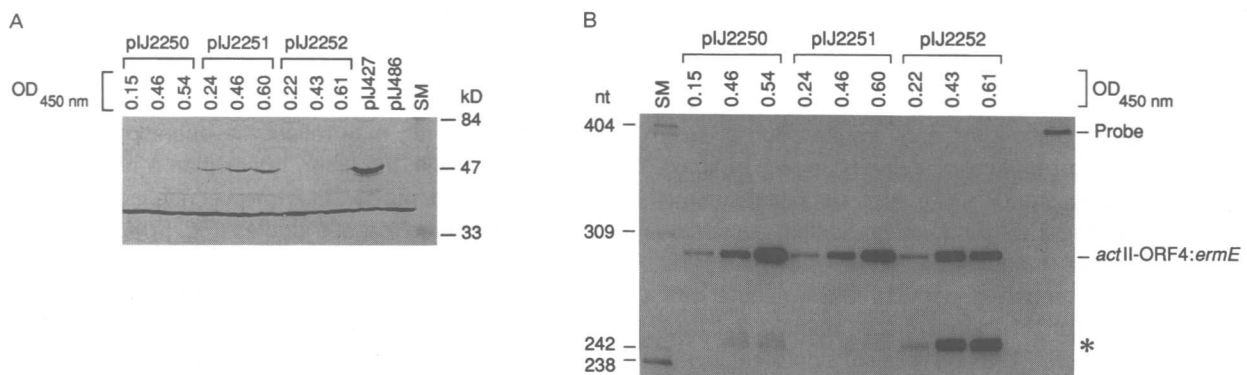


Fig. 6. A. Western analysis of cell extracts of *S. coelicolor* A3(2) strain J1700 derivatives containing pIJ2250, pIJ2251 and pIJ2252, using antibodies against the *ermE* methylase. Cell extracts from J1700 containing pIJ427 and pIJ486 were used as positive and negative controls, respectively; SM, size markers.

B. S1 nuclease mapping of *actII-ORF4::ermE* transcripts in RNA samples from the same cultures used in (A). Probe, the location of full-length probe. See the legend to Fig. 5B for other abbreviations.

conditions used, is the availability of enough of the activator. Although the elevated levels of the *actII*-ORF4 transcript obtained with pIJ68 might reflect titration of a putative *actII*-ORF4 repressor, there is no genetic evidence to suggest the existence of a negatively acting regulatory gene. The reason for the increase in the level of the *actII*-ORF4 mRNA, and presumably therefore the *actIII* transcript, during early exponential growth of M145(pIJ68) (Fig. 2) and the continual rise in the level of the fusion transcripts throughout exponential growth (Figs. 5B and 6B) is not known, but may reflect increases in copy number of pIJ101 derivatives such as pIJ486 during rapid growth (H. Richards, personal communication). Considerably higher levels of *actII*-ORF4::*ermE* mRNA were observed for pIJ2252, relative to pIJ2250 and pIJ2251, in both M145 and J1700. This may reflect a more stable transcript, but why this should result from the presence of three consecutive UUA codons is obscure; sequence analysis failed to reveal any potential secondary structure in this region of the fusion transcripts that might have influenced mRNA stability.

The pleiotropic effect of *bldA* mutations on antibiotic production, the unusual distribution of UUA codons and data suggesting that *bldA*, in surface-grown cultures, was transcribed only later in growth (Lawlor *et al.*, 1987), led to the proposal that *bldA* represented a novel mechanism of translational control of antibiotic production (Chater, 1990; Leskiw *et al.*, 1991b). This was consistent with data indicating that the transcription of undecylprodigiosin (Guthrie and Chater, 1990), actinorhodin (Bruton *et al.*, 1991; Fernández-Moreno *et al.*, 1991) and methylenomycin (A. Wietzorrek and K. F. Chater, personal communication) biosynthetic structural genes was greatly reduced in *bldA* mutants. In the conditions we generally use, which were developed to analyse the growth-phase-dependent expression of antibiotic biosynthetic genes in liquid culture, abundant amounts of a transcript corresponding to the 5' end of the mature *bldA* tRNA were detected in young, exponentially growing cultures (Fig. 3). Since essentially identical results were obtained using richer medium and spores that had not been germinated prior to inoculation, the early transcription of *bldA* may be a common feature of cultures grown in liquid medium. Moreover, the tRNA^{Leu}_{UUA} was functional since it could translate the UUA codons present in the *actII*-ORF4::*ermE* fusion transcripts in equally young cultures, and there was no apparent increase in the relative efficiency of translation of UUA codons, relative to UUG codons, during growth. The possibility of significant levels of mis-translation of the UUA codons in the fusion mRNAs was ruled out by the virtual absence of the fusion protein in the *bldA* mutant J1700 containing either pIJ2250 or pIJ2252 (the low level of fusion protein detected at the last time point for the later construct (Fig. 6A) may reflect

a low level of mis-translation of the relatively abundant transcript). These observations suggest that *bldA* does not play a role in the temporal regulation of *actII*-ORF4 expression and actinorhodin production in the culture conditions used here.

In contrast to the results reported here, B. Leskiw and co-workers (personal communication) have obtained data that are consistent with the temporal activation of *bldA*; at present we have no explanation for the disparity between the two sets of results. Although we have not replaced the UUA codon of the chromosomal *actII*-ORF4 with a synonymous leucine codon and assessed the effect on the timing of actinorhodin production, the low level of *actII*-ORF4 transcription during exponential phase, and the lack of any detectable difference in the apparent efficiency of translation of UUA and UUG codons throughout growth, strongly suggest that such a change would have no effect.

The incidence of UUA codons in *Streptomyces* genes remains intriguing. With a few exceptions (for which possible explanations have been proposed), UUA codons are found only in genes that one would expect to be expressed only late in the growth of the organism (Leskiw *et al.*, 1991b), consistent with a potential regulatory role for the codons. But if UUA codons are not regulatory targets for *bldA*, how can their incidence be explained? Presumably, during evolution, streptomycetes have been subjected to a mutational bias that has led to a high genomic G+C content (on average, 74 mol%; Gladek and Zakrzewska, 1984) and consequently to a high degree of synonymous codon preference in which G+C-rich codons predominate (Wright and Bibb, 1992). In addition to mutational bias, there is evidence for translational selection in *Streptomyces* (Wright and Bibb, 1992); if UUA codons were inefficiently translated, they might have been selectively removed from genes expressed at high and moderate levels, but retained in those expressed at low levels. Regulatory genes, like *actII*-ORF4, are generally expected to be expressed at low levels. The occurrence of UUA codons in such genes, and not in the primary metabolic and antibiotic biosynthetic structural genes sequenced so far, may reflect the operation of translational selection in the latter, rather than a novel mechanism of translational control. However, the levels of fusion proteins observed in these studies (Fig. 5) would suggest that UUA is translated at least as efficiently as UUG. Furthermore, the correspondence between the amount of fusion transcript and protein observed with M145/pIJ2251 and M145/pIJ2252 suggests that the presence of UUA codons is not a rate-limiting factor during translation. Thus, in the absence of a confirmed regulatory role for *bldA*, a satisfactory explanation for the unusual occurrence of UUA codons remains elusive.

From these studies we conclude that the growth-phase-

dependent regulation of actinorhodin biosynthesis, under the conditions used, occurs through the transcriptional control of the activator gene *actII-ORF4*. Similar results have been obtained for the regulation of undecylprodigiosin in the same strain (Takano *et al.*, 1992), where the expression of the pathway-specific activator gene, *redD*, is also transcriptionally regulated, and shows a strong growth-phase dependence. How the transcriptional control of activator genes such as *redD* and *actII-ORF4* occurs must now be addressed, and it is reasonable to assume that at least some of the previously recognized pleiotropic genes play a direct role in their activation.

Experimental procedures

Bacterial strains and plasmids

Escherichia coli strains were JM101 (Sambrook *et al.*, 1989) and ET12567 (F⁻, *dam*-13::Tn9, *dcm*-6, *hsdM*, *hsdR*, *zji*-202::Tn10, *recF*143, *galK*2, *galT*22, *ara*-14, *lacY*1, *xyl*-5, *leuB*6, *thi*-1, *tonA*31, *rpsL*136, *hisG*4, *tsx*-78, *mtl*-1, *glnV*44; a gift from Tanya MacNeil). *S. coelicolor* A3(2) strains were M145 (SCP1⁻, SCP2⁻, prototrophic; Hopwood *et al.*, 1985), J1501 (*hisA*1, *uraA*1, *strA*1, *pgl*, SCP1⁻, SCP2⁻; Hopwood *et al.*, 1985), and its derivatives, J1700 (*bldA*39; Leskiw *et al.*, 1991b; Lawlor *et al.*, 1987) and J1681 (Δ *bldA*; B. K. Leskiw, personal communication). *E. coli* plasmid pIJ452 (pUC18 (Yanisch-Perron *et al.*, 1985) containing the 1.4 kb *Bam*HI–*Kpn*I *ermE* coding region (Bibb *et al.*, 1985)) was used as a source of *ermE* for the construction of the translational fusions, and pIJ5104 (Hallam *et al.*, 1988) and pIJ2334 (a pBR329 derivative carrying *act* DNA between sites 10 and 13 of the restriction map of Malpartida and Hopwood (1986)) were used as sources of probes for S1 nuclease mapping of *actIII* and *actII-ORF4*, respectively. *Streptomyces* plasmids pIJ2303 (Malpartida and Hopwood, 1986) and pIJ580 (Lawlor, 1987) were used as templates for the polymerase chain reaction (PCR) amplification (Erich, 1989) of parts of *actVI-ORF1* and *bldA*, respectively; pIJ68 (Passantino *et al.*, 1991) is a derivative of the multicopy vector pIJ699 (Kieser and Melton, 1988) containing *actII-ORF4*, pIJ486 (Ward *et al.*, 1986) was used as vector for the *actII-ORF4::ermE* fusions, and pIJ427 is pIJ486 containing *ermE* as a 1.7 kb *Kpn*I fragment (Bibb *et al.*, 1985), with *ermE* transcribed in the same direction as the *neo* gene of the vector.

Culture conditions and microbiological procedures

Liquid cultures of *S. coelicolor* A3(2) were grown in a minimal medium supplemented with 0.2% w/v casamino acids (Takano *et al.*, 1992), and actinorhodin was detected as described by Strauch *et al.* (1991); protoplast production and transformation were as in Hopwood *et al.* (1985). *E. coli* strains were grown and transformed by standard procedures (Sambrook *et al.*, 1989).

Manipulation of DNA, RNA and cloning procedures

Standard procedures were used for *S. coelicolor* A3(2) (Hopwood *et al.*, 1985) and for *E. coli* (Sambrook *et al.*, 1989).

Construction of the *actII-ORF4::ermE* translational fusions

The PCR was used for mutagenesis of the sequence containing the *actII-ORF4* promoter and 5' coding region. The 5' oligonucleotide was 5'-AAGCTTGGATCCTCGCTGCACTGATTAATT-3' with a *Bam*HI site located at positions 7–12. The sequences of the 3' oligonucleotides were complementary to those shown in Fig. 4; all contained a *Bam*HI site that allowed the PCR products to be cloned into *Bam*HI-cleaved pIJ452 such that the *N*-terminal coding region of *actII-ORF4* was in frame with the full-length *ermE* gene present in the vector. JM101 transformants were selected on Luria–Bertani (LB) plates containing ampicillin (100 µg ml⁻¹) and then replica-plated onto LB plates containing ampicillin (100 µg ml⁻¹) and lincomycin (300 µg ml⁻¹) to obtain those with the *actII-ORF4* segment in the correct orientation (previous work had indicated that protein fusions could be made to the *N*-terminus of the methylase that resulted in an enzyme that could still confer resistance to lincomycin; M. J. Bibb, unpublished). *Xba*I–*Sst*I fragments containing the *actII-ORF4::ermE* fusions were gel-purified and cloned into the *Streptomyces* multicopy vector pIJ486 cleaved with the same enzymes; *S. coelicolor* M145 transformants were selected on thioestrepton (200 µg ml⁻¹) and then replica-plated to lincomycin (500 µg ml⁻¹). The predicted sequences of the final constructions were confirmed using the dideoxy-chain termination method (Sanger *et al.*, 1977) adapted for use with plasmid DNA (G. Murphy, personal communication) and the modified T7 DNA polymerase (Sequenase, USB) using oligonucleotide primers synthesized on a Pharmacia Gene Assembler.

S1 nuclease mapping

For each S1 nuclease reaction, 10–30 µg of RNA were hybridized in NaTCA buffer (Murray, 1986) to about 0.02 pmol (approximately 10⁴ Cerenkov counts min⁻¹) of the following probes. For *actII-ORF4* (Fernández-Moreno *et al.*, 1991), a 465 bp *Xho*I–*Asel* fragment containing the *actII-ORF4* promoter region uniquely labelled at the 5' end of the *Xho*I site within the *actII-ORF4* coding region was used. For *actIII* (Hallam *et al.*, 1988), a 360 bp *Sal*I–*Bam*HI fragment was used that contained the *actIII* promoter region uniquely labelled at the 5' end of the *Sal*I site within the *actIII* coding region. For *bldA*, the 5' end of the synthetic oligonucleotide 5'-CGGAGCCG-GACTTGAACC-3', corresponding to a region close to the end of the *bldA* gene (Lawlor *et al.*, 1987), was labelled with [³²P]-ATP using T4 polynucleotide kinase, and then used in the PCR with the unlabelled 5' oligonucleotide 5'-CATGGATCCACC-CGGTAACTGATGCACC-3' that corresponds to the region upstream of the *bldA* promoter to generate a 206 bp probe. For *actVI-ORF1* (F. Malpartida, personal communication), the 5'-labelled 3' oligonucleotide 5'-ACGTCCGGCTCGTACTC-GATG-3' and the unlabelled 5' oligonucleotide 5'-CTTGCG-GTGGAAAGTCCCTCCAG-3' (whose sequence lies an undetermined distance upstream of the *actVI-ORF1* promoter) were used in a similar fashion to generate a 471 bp probe. For the *actII-ORF4::ermE* transcripts, the 5' end of the labelled oligonucleotide 5'-GAGTTCCTGGTGAGCAGCCCTTC-3' (corresponding to a region approximately 250 bp downstream of the *ermE* GTG start codon; Bibb *et al.*, 1985), was used in

the PCR with the unlabelled oligonucleotide 5'-AAGCT-TGGATCCTCGCTGCACTGATTATT-3' (corresponding to sequences upstream of the *actII*-ORF4 promoter) and pJ2250 template DNA to generate a 403 bp probe. All subsequent steps were as described by Strauch *et al.* (1991). For quantitative estimates of the relative amounts of the RNA-protected fragments, X-ray films were preflashed, exposed at -70°C (Laskey, 1980) and scanned on a Joyce-Loebl Chromoscan 3 scanning densitometer. Increasing the amount of RNA in step-wise fashion from 5 μg to 50 μg led to a proportional increase in the level of protected fragment, indicating conditions of probe excess for all of the S1 mapping experiments reported.

Protein extraction and Western blotting

Cell extracts were prepared as previously described (Gramajo *et al.*, 1991) and protein concentrations were determined by the method of Bradford (1976); fractions were analysed by SDS-PAGE (Laemmli, 1970) and by Western blotting (Burnette, 1991). Antibodies against the *N*-terminus of the *ermE* methylase were raised in rabbits using the synthetic oligopeptide MSSSDEQPRPRRR (Bibb *et al.*, 1985). The antibodies were used at 1:4000 dilution with a goat anti-rabbit IgG-alkaline phosphatase conjugate (Bethesda Research Laboratories) as second antibody. Relative amounts of fusion protein were determined by scanning negatives of developed blots on a Joyce-Loebl Chromoscan 3 scanning densitometer; varying the amount of cell extract loaded onto the gel confirmed that the amounts of fusion protein quantified fell within the linear range of the negative and densitometer.

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